Oxime-induced Reactivation of Carboxylesterase Inhibited by Organophosphorus Compounds

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ABSTRACT

A structure-activity analysis of the ability of oximes to reactivate rat plasma carboxylesterase (CaE) that was inhibited by organophosphorus (OP) compounds revealed that uncharged oximes, such as diacetylmonoxime or monoisonitrosoacetone, were better reactivators than cationic oximes. Cationic oximes that are excellent reactivators of OP-inhibited acetylcholinesterase, such as pyridinium-2-aldoxime or the bis-pyridinium oximes, HI-6 and TMB-4, produced poor reactivation of OP-inhibited CaE. The best uncharged reactivator was diacetylmonoxime which produced complete reactivation at 0.3 mM in 2 hr of CaE that was inhibited by organophosphinates, alkoxy-containing phosphates, and alkoxy-containing phosphonates. Complete reactivation of CaE could be achieved even after inhibition by phosphonates with highly branched alkoxy groups, such as sarin and soman, that undergo rapid aging with acetylcholinesterase. CaE that was inhibited by phosphonates or phosphates that contained aryloxy groups were reactivated to a lower extent. The cause of this decreased reactivation appears to be an oxime-induced aging reaction that competes with the reactivation reaction. This oxime-induced aging reaction is accelerated by electron-withdrawing substituents on the aryloxy groups of phosphonates and by the presence of multiple aryloxy groups on phosphates. Thus, reactivation and aging of OP-inhibited CaE differ from the same processes for OP-inhibited acetylcholinesterase in both their oxime specificity and inhibitor specificity and, presumably, in their underlying mechanisms.

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INTRODUCTION

Organophosphorus (OP) compounds can inhibit a variety of serine esterases. Among these esterases are acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme that terminates the action of acetylcholine in the nervous system, and carboxylesterase (CaE; EC 3.1.1.1) an enzyme that detoxifies OP compounds (Maxwell, 1992a). A variety of oximes have been synthesized that can reactivate OP-inhibited esterases (Wilson et al., 1992). However, some OP-inhibited esterases become refractory to oxime reactivation because of "aging", a dealkylation process that results in an OP-inhibited esterase containing a P-O⁻ group that resists reactivation (Michel et al., 1967; Harel et al., 1991). Oxime reactivation and aging of OP-inhibited AChE has been extensively studied and effective cationic oximes, such as 2-PAM, TMB-4 and HI-6, have been adopted as medical treatment for OP poisoning (Taylor et al., 1985). Oxime reactivation of OP-inhibited CaE has been studied in only a few papers (Myers, 1959; Sterri et al., 1983; De Jong and Wolring, 1984; Bryson and Brown, 1985) and no structure-activity analysis for oxime reactivation of OP-inhibited CaE has been reported.

The purpose of this study was (1) to measure the in vitro oxime-induced reactivation of CaE that had been inhibited by a series of OP compounds (See Table I for structures), (2) to evaluate the ability of several types of oximes to reactivate OP-inhibited CaE, and (3) to examine the aging of OP-inhibited CaE.

METHODS AND MATERIALS

Chemicals. Compounds I, VI, VII, VIII, IX were obtained from Chemical Research, Development and Engineering Center (Aberdeen Proving Ground, MD). Compounds II and XVII were purchased from Pfaltz and Bauer (Waterbury CT). Compounds III, IV, V, X, XI, XII, and XIII were synthesized the methods of Hovanec and Lieske (1972) and Lieske et al. (1980). Compounds XIV, XV, XVI as well as DAM, INAP, and TMB-4 were purchased from Aldrich Chemical (Milwaukee, WI). PAM and HI-6 were obtained from the Defense Research Establishment (Suffield, Canada).

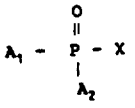
Enzyme preparation. CaE was enriched from rat plasma (Pel-Freez, Rogers, AR) by gel filtration on Sephadex G-200 by the method of Hashinotsume et al. (1978).

Enzyme analysis. CaE was assayed by the spectrophotometric method of Ecobichon and Comeau (1973) with 1-naphthyl acetate as substrate.

CaE inhibition and reactivation. Inhibition of CaE was performed in 0.05 M phosphate buffer (pH 7.4) at 25 °C with solutions of each OP compound in acetonitrile. Twenty microliters of inhibitor solution were added to 1.00 ml of phosphate buffer containing 3 nanomoles of CaE. The CaE

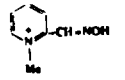
Table I

Structures of Organophosphorus Compounds

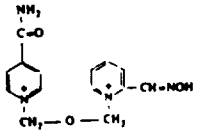


Organophosphorus Compound	A_1	A_2	X
Instant Aging			
I	Me	Cl	Cl
II	Ph	Cl	Cl
Phosphinates			
III	Me	Me	$OPh\text{-}4\text{-}NO_2$
IV	Me	Ph	$OPh\text{-}4\text{-}NO_2$
V	Ph	Ph	$OPh\text{-}4\text{-}NO_2$
Phosphonates			
VI (VX)	Me	OEt	$SC_2H_4N(Pr^i)_2$
VII (Sarin)	Me	OPr^i	F
VIII (Soman)	Me	OPin	F
IX (GF)	Me	$OHex^c$	F
X	Me	OPh	Cl
XI	Me	OPh-4-OMe	Cl
XII	Me	$OPh\text{-}4\text{-}NO_2$	$OPh\text{-}4\text{-}NO_2$
XIII	Me	OPh-4-CN	OPh-4-CN
Phosphates			
XIV (Dichlorvos)	OMe	OMe	$OCH{=}Cl_2$
XV (Paraoxon)	OEt	OEt	$OPh\text{-}4\text{-}NO_2$
XVI (DFP)	OPr^i	OPr^i	F
XVII	OPh	OPh	Cl

Structures of Oximes

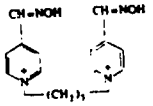


2-PAM



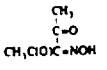
HI-6

Isonitroso acetophenone (INAP)



TMB-4

Monoisonitrosoacetone (MINA)



Diacetyl monoxime (DAM)

activity in the reaction mixture was measured after a 10-min incubation to confirm that >95% inhibition of CaE had occurred. The OP-inhibited CaE was then separated from excess inhibitor by high performance liquid chromatography by injection of 100 ul of the reaction mixture onto a TSK-gel SW2000 column equilibrated with phosphate buffer. With a column flow rate of 1.3 ml/min, OP-inhibited CaE eluted in the column fractions collected between 9 and 10 ml after sample injection, where control CaE samples were previously found to elute. Each OP-inhibited CaE sample was compared to a corresponding control CaE sample which had received identical incubation and chromatography treatment except for the presence of OP inhibitor. After chromatographic separation the sample of OP-inhibited CaE (1,300 ul volume) was reactivated at 25°C by addition of 650 ul of 10^{-3} M oxime in 0.05 M phosphate (pH 8.0). Samples of the oxime reaction mixture (300 ul) were assayed sequentially for CaE activity at time intervals up to 2 hr.

The observed rate constant (k_{obs}) for oxime reactivation of OP-inhibited CaE was calculated from the velocity of CaE activity (v) observed at sequential times (t) after the addition of oxime using the equation: $v = V_{max}(1-e^{-k_{obs}t})$ where V_{max} was the maximal recovery of CaE activity. Inasmuch as the oxime-induced reactivation and aging of OP-inhibited enzymes are parallel first-order reactions, the true rate constants for oxime-induced reactivation (k_r) and aging (k_a) were calculated similar to the method of Hovanec and Lieske (1972).

RESULTS

The ability of a range of commonly used oximes (See Table I for structures) to reactivate OP-inhibited CaE is shown in Table II. Cationic oximes were uniformly poor in their reactivation of OP-inhibited CaE, regardless of the structures of the OP compound. Uncharged oximes were much better reactivators of OP-inhibited CaE than cationic oximes. Among the uncharged oximes the ability to reactivate OP-inhibited CaE decreased in the order DAM > MINA > INAP. For studies of oxime-induced reactivation the only substituents of OP inhibitors that are of interest are A_1 and A_2 since X is cleaved from the OP compound when it inhibits CaE. The best reactivator, DAM, was able to reactivate completely all OP compounds except for those containing a chloro substituent in the A_2 position (I and II), which exhibited no reactivation, and compounds containing a phenoxy group (X - XIII, XVII) which exhibited < 55% reactivation. The compounds containing a chloro substituent in position A_2 (I, II) were used as negative controls for oxime-induced reactivation since P-Cl bonds are known to hydrolyze readily, leaving a P-O$^-$ inhibiting moiety which is not reactivated (i.e., instant aging). The phosphinate inhibitors (III-V) were used as positive controls for reactivation since

Table II

Oxime Reactivation of Carboxylesterase Inhibited by Organophosphorus Compounds

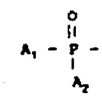
Organophosphorus Compounds	% Reactivation[a]					
	Uncharged Oximes			Cationic Oximes		
	MINA	DAM	INAP	2-PAM	TMB-4	HI-6
Irstant Aging						
I[b]	0	0	0	0	0	0
II[b]	0	0	0	0	0	0
Phosphinates						
III	69±3	99±3	22±3	13±4	8±3	0
IV	66±4	99±2	86±5	31±3	42±2	35±3
V	66±3	96±4	90±4	12±3	20±1	20±4
Phosphonates						
VI (VX)[b]	96±4	98±2	45±6	6±4	9±3	5±3
VII (Sarin)	85±6	99±3	15±4	0	0	0
VIII (Soman)	68±5	97±4	0	0	0	0
IX (GF)	74±4	96±4	0	0	0	0
X[b]	40±4	45±5	0	0	0	b
XI[b]	23±3	55±2	0	0	0	0
XII	19±4	9±2	0	0	0	0
XIII	0	15±3	0	0	0	0
Phosphates						
XIV (Dichlorvos)	75±7	95±5	20±6	0	0	0
XV (Paraoxon)	64±5	98±3	0	0	0	0
XVI (DFP)	71±4	97±5	0	0	0	0
XVII[b]	4±1	5±1	0	0	0	0

a. 2 hr reactivation with 0.3 mM oxime at 25°C (pH 7.4).
% reactivation expressed as means ± S.D. (n=3).

b. Required 10^{-3}M concentration for inhibition of CaE, while all other OP compounds required 10^{-4}M.

Table III

Aging and Reactivation by Diacetylmonoxime of Carboxylesterase Inhibited by Organophosphorus Compounds



Organophosphorus Compound	Inhibited Species		Rate Constant (hr^{-1})[a]		
	A_1	A_2	k_{obs}	k_r	k_a
Instant Aging					
I	Me	Cl	0	-	-
II	Ph	Cl	0	-	-
Phosphinates					
III	Me	Me	1.4±0.1	1.4±0.1	0
IV	Me	Ph	1.3±0.2	1.3±0.2	0
V	Ph	Ph	1.4±0.3	1.4±0.2	0
Phosphonates					
VI (VX)	Me	OEt	1.3±0.2	1.3±0.2	0
VII (Sarin)	Me	OPr^i	1.4±0.2	1.4±0.2	0
VIII (Soman)	Me	OPin	1.3±0.1	1.3±0.1	0
IX (GF)	Me	$OHex^c$	1.5±0.3	1.5±0.3	0
X	Me	OPh	2.9±0.2	1.3±0.1	1.6±0.1
XI	Me	OPh-4-OMe	2.5±0.2	1.4±0.1	1.1±0.1
XII	Me	OPh-4-NO_2	16.7±2.2	1.5±0.2	15.2±2.0
XIII	Me	OPh-4-CN	9.3±1.1	1.4±0.2	7.9±0.9
Phosphates					
XIV (Dichlorvos)	OMe	OMe	0.8±0.1	0.8±0.1[b]	0
XV (Paraoxon)	OEt	OEt	1.0±0.2	1.0±0.2[b]	0
XVI (DFP)	OPr^i	OPr^i	0.7±0.1	0.7±0.1[b]	0
XVII	OPh	OPh	26.0±2.3	0.9±0.1[b]	17.1±2.2

a. Reactivation with 0.3 mM DAM at 25°C (pH 7.4).
Rate constants are expressed as means ± S.D.

b. Significant difference from phosphinates and phosphonates.

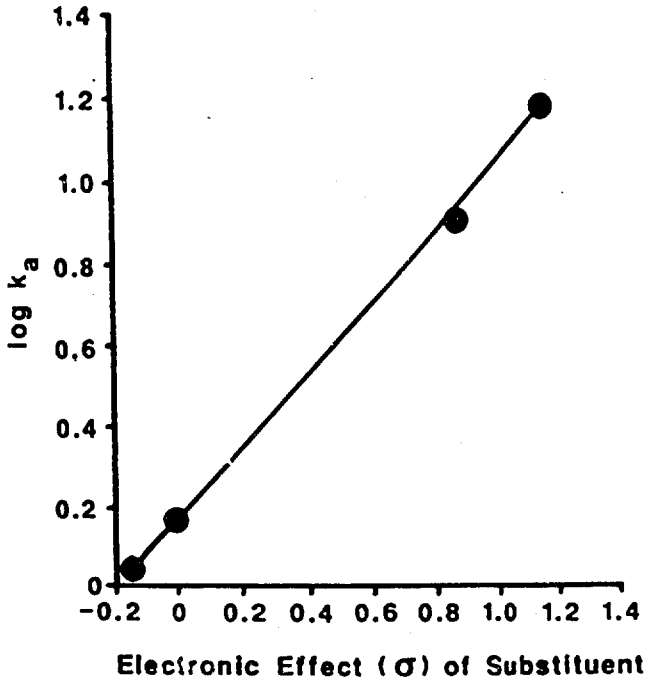


Figure 1. Effect of electronic parameter (σ) of aryloxy substituent on aging rate (k_a) of CaE inhibited by organophosphorus compounds. Substituents are presented in order of increasing σ for inhibited species Me(OPhX)P(0)-CaE where X = H, OMe, CN or NO_2. Data for σ from Hansch and Leo (1970).

they contain A_1 and A_2 substituents that have P-C bonds, which are not subject to dealkylation (i.e., aging) and are, therefore, capable of complete oxime-induced reactivation.

Compounds VI-XIII are organophosphonates that contain one P-O-C bond that is susceptible to aging. Compounds XIV-XVII are phosphates which contain two P-O-C bonds that are also susceptible to aging. The influence of the structure of OP moieties on the oxime reactivation of OP-inhibited CaE was evaluated by measuring the observed rate constants of oxime-induced reactivation (k_{obs}) by DAM, the best reactivator of OP-inhibited CaE. The observed rates of oxime-induced reactivation were separated into their constituent rate constants of reactivation (k_r) and aging (k_a) which are shown in Table III. As expected, compounds I and II, which have structures consistent with instant aging, have no observable oxime-induced reactivation; and compounds III-V, which have structures that cannot age, have complete oxime-induced reactivation with DAM. The rate constants for reactivation of different phosphinate-inhibited CaE were statistically indistinguishable (1.3 to 1.4 hr^{-1}).

CaE that was inhibited by phosphonates had rate constants of oxime reactivation (k_r) that varied in a narrow range from 1.3 to 1.5 hr^{-1}, which was similar to k_r for phosphinates. The alkoxy-containing phosphonates (VI-VX) exhibited no aging while the aryloxy-containing phosphonates (X-XIII) exhibited a wide variation in rate constants of aging, ranging from 1.6 to 15.2 hr^{-1}. The aging rate for the aryloxy phosphonates appears to be related to the electronic parameter (σ), which is an estimate of the ability of a substituent on an aryl ring to withdraw ($+\sigma$) or donate ($-\sigma$) electrons to the ring. Fig. 1 shows the linear relationship between σ and the rate constants of aging for the H, OMe, CN and NO_2 substituents of aryloxy-containing phosphonate inhibitors of CaE.

CaE that was inhibited by phosphates had rate constants of oxime reactivation that varied in a narrow range from 0.7 to 1.0 hr^{-1} which was about 2/3 of the values of k_r for phosphinates and phosphonates. The alkoxy-containing phosphates (XIV-XVI) exhibited no aging, while the sole aryloxy-containing phosphate (XVII) had a rate constant of aging of 17.1 hr^{-1}, which was 20 times larger than its reactivation rate constant.

DISCUSSION

Oxime-induced reactivation and aging of OP-inhibited CaE has several characteristics that distinguish it from the more extensively studied oxime-induced reactivation and aging of OP-inhibited AChE. First, the oximes that are effective reactivators of OP-inhibited CaE -- MINA, INAP, and DAM -- are all uncharged oximes, while the most ineffective oximes -- PAM, TMB-4, and HI-6 -- are all cationic oximes (Table II). This

pattern of oxime effectiveness is quite different from the pattern of oxime reactivation for OP-inhibited AChE, where cationic oximes are far superior to uncharged oximes (Wilson et al., 1992). This phenomenon is probably related to the differences in the substrate and inhibitor specificities of these two enzymes. AChE has a strong specificity for cationic substrates (e.g., acetylcholine) and inhibitors (e.g., VX) resulting in the hypothesis that it contains an anionic center in its active site (Taylor, 1985). In contrast, CaE does not react well with acetylcholine or VX or a variety of other charged substrates and inhibitors, but it has a broad specificity for uncharged substrates and inhibitors (Maxwell, 1992a,b). Second, although the aging of OP-inhibited CaE and AChE share some similarities, the aging process in these enzymes appears to occur by different mechanisms. Both CaE and AChE that have been inhibited by instant aging compounds, such as I and II, are not susceptible to oxime reactivation, and both CaE and AChE that have been inhibited by phosphinates are completely reactivated by effective oximes. However, the structure-activity patterns of aging for CaE and AChE that have been inhibited by phosphonates and phosphates are completely different. The most rapidly aging OP compounds for AChE contain highly branched alkoxy substituents, such as isopropoxy (e.g., sarin, DFP) or pinacoloxy (e.g., soman), which can stabilize the formation of a carbonium ion that is believed to be produced during the aging process (Michel et al., 1967; Harel et al., 1991). OP compounds that contain these highly branched alkoxy substituents do not age after their inhibition of CaE. Therefore, the mechanism of aging of OP-inhibited CaE probably does not proceed through a carbonium ion mechanism.

The structural characteristic of OP compounds that was most related to the aging of OP-inhibited CaE was the pK_a of the acid formed by the hydrolysis of P-O-C bonds. The OP compounds that aged contained aryloxy groups while the compounds that did not age contained alkoxy groups (Table III). The pK_a of alkoxy groups is > 14 while the pK_a of aryloxy groups is $\leq$ 10 (Dean, 1985). The lower pK_a of the aryloxy groups suggests that the P-O-C bonds of OP compounds containing this group are more labile than the corresponding bonds for alkoxy groups. This argument could be extended to substituents on phenoxy groups as well as differences between alkoxy and aryloxy groups. Electron withdrawing substituents ($+\sigma$), such as CN or NO_2, on phenoxy-containing phosphonates (i.e., XII and XIII) increased the aging rate constant in comparison to unsubstituted phenoxy phosphonates, while electron donating substituents, such as OMe, decreased the aging rate constant (Fig. 1). This type of oxime-induced aging has previously been described for OP-inhibited chymotrypsin (Erlanger, 1967; Toia and Casida, 1979; Harel et al., 1991), which also does not age by the classical carbonium ion mechanism of OP-inhibited AChE.

The most significant practical finding of this research was

that CaE that has been inhibited by alkoxy-containing OP compounds, which constitute most of the commercial and military OP compounds, can be rapidly and completely reactivated. The only OP compounds that undergo aging after reaction with CaE are the aryloxy-containing OP compounds. Thus, endogenous CaE, which is an important stoichiometric detoxication enzyme for OP compounds (Maxwell, 1992a), when in the presence of an uncharged oxime, becomes even more effective because it is easily reactivated for further stoichiometric removal of most OP compounds. To put the magnitude of these rates of CaE reactivation into perspective, the rates of DAM reactivation of VX-, sarin-, or soman-inhibited CaE were approximately equal to the rates of TMB-4 reactivation of AChE and about 1/5 the rates of HI-6 reactivation of AChE that has been inhibited by these same OP compounds (Skrinjaric-Spoljar and Kral, 1980; Su et al., 1986; De Jong and Wolring, 1984). Therefore, oxime-induced reactivation of OP-inhibited CaE for protection by enhancement of OP detoxication occurs at approximately the same rate as oxime-induced reactivation of OP-inhibited AChE for protection by pharmacological intervention at cholinergic synapses.

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